

Synthesis of gold nanoparticles by the fungus *Aspergillus niger* and its efficacy against mosquito larvae

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Background: The fungus *Aspergillus niger* has been selected for the synthesis of gold nanoparticles (AuNPs). The authors report the role of fungi in the synthesis of AuNPs. Additionally, the larvicidal efficacy of these AuNPs was tested using the larvae of three mosquito species: *Anopheles stephensi*, *Culex quinquefasciatus*, and *Aedes aegypti*.

Methods: The AuNPs were characterized through microplate reader analysis, X-ray diffraction, transmission electron microscopy, and scanning electron microscopy. The larvicidal efficacy was tested against all larval stages of *A. stephensi*, *C. quinquefasciatus*, and *A. aegypti* and calculated by probit analysis at six different log concentrations at the time points of 24, 48, and 72 hours.

Results: The AuNPs synthesized by *A. niger* were found to be more effective against the *C. quinquefasciatus* larvae than the *A. stephensi* and *A. aegypti* larvae. All larval instars of *C. quinquefasciatus* showed 100% mortality after 48 hours of exposure to the AuNPs synthesized by *A. niger*.

Conclusion: The results suggest that the use of AuNPs synthesized by fungus can be a more rapid and environmentally friendly approach for mosquito control than current approaches. This could potentially lead to a new vector control strategy.

Keywords: nanoparticles, biosynthesis, fungi, larvicide, mosquito-borne disease

Introduction

Mosquitoes are responsible for spreading serious diseases such as malaria, dengue fever, yellow fever, Japanese encephalitis, Chikungunya fever, and so forth. *Anopheles* spp. are the most important, as they are capable of carrying malaria parasites. Approximately half of the world's population is at risk of malaria, particularly those living in lower-income countries. Malaria infects more than 500 million people per year and kills more than one million.¹ "Mosquito control" is the control of mosquito-borne diseases through the interruption of disease transmission by killing or preventing mosquitoes from biting humans. Mosquito control is a vital public health practice throughout the world, especially in the tropics. It is known that larvicides play a vital role in controlling mosquitoes in mosquito breeding sites. However, the development of resistance to larvicides in various mosquito populations has, unfortunately, also been reported.

For people to be protected from mosquito-borne diseases, it is essential that the mosquito population is controlled. Biological control, used as an alternative to current larvicides for minimizing the mosquito population, can provide an effective and environmentally friendly approach to mosquito control. Currently, fungi are being utilized in nanotechnology for the production of nanoparticles; synthesis using fungi has shown

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that this environmentally benign and renewable source can be used as an effective reducing agent for synthesis of silver nanoparticles (AgNPs) and gold nanoparticles (AuNPs). This biological reduction of metal could be utilized for a clean, nontoxic, and environmentally acceptable “green” approach to producing metal nanoparticles. It is well known that some microbes such as bacteria,² yeast,³ and fungi⁴ are potentially useful in the preparation of metal nanoparticles under normal air pressure and at room temperature. Many species of fungi have been used in nanotechnology for nanoparticle production, including *Fusarium oxysporum*,⁵ *Aspergillus fumigatus*,⁶ *Verticillium* spp.,⁷ and *Chrysosporium tropicum*.⁸ The AgNPs and AuNPs formed are highly stable and have significant mosquito larvicidal activity. The antiparasitic activities and efficacy of AgNPs synthesized using aqueous leaf extract of *Mimosa pudica* have been evaluated against the larvae of malaria vector *Anopheles subpictus*, filariasis vector *Culex quinquefasciatus*, and *Rhipicephalus microplus*.⁹ In the present article the authors describe the larvicidal effect of extracellular synthesis of AuNPs with *Aspergillus niger* – the problem of resistance is effectively minimized using a new fungal-based nanolarvicide.

Material and methods

Fungal strain

The fungal strain of *A. niger* (MTCC 2587) was obtained from the Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India, and was routinely maintained in the laboratory on Czapek-Dox agar at 25°C.

Preparation of broth and culture of *A. niger*

The broth was prepared for culture of *A. niger* following the method of Gardner and Pillai.¹⁰ Five 250 mL conical flasks, each containing 100 mL of Czapek-Dox broth (sucrose 30 g, sodium nitrate 3 g, dipotassium phosphate 1 g, magnesium sulfate 0.05 g, potassium chloride 0.05 g, ferrous sulfate 0.01 g, and deionized water 1000 mL), were autoclaved at 20 psi for 20 minutes. The broth was supplemented with chloramphenicol (50 µg/mL) as a bacteriostatic agent. *A. niger* colonies grown on the Czapek-Dox agar plates were transferred to each flask by inoculation needle. The conical flasks inoculated with *A. niger* were incubated at 25°C for 15 days.

Collection and maintenance of mosquito larvae

Mosquito larvae were collected from various localities including urban, rural, and semi-urban regions of Agra

(northern India, 27°10'N, 78°05'E) and they were reared in deionized water containing glucose and yeast powder. The *Anopheles stephensi*, *C. quinquefasciatus*, and *Aedes aegypti* colonies were maintained in the laboratory on 14-hour photoperiods at 25°C, with a relative humidity of 75% plus or minus 5%. The *A. stephensi*, *C. quinquefasciatus*, and *A. aegypti* larvae were maintained in separate enamel containers, as per the standard method.¹¹

Synthesis of AuNPs

After incubation the fungal biomass was separated from the medium by filtration through filter paper (grade 1; Whatman plc, Maidstone, Kent, UK) and washed three times in sterile distilled water to remove any nutrient media that could interact with the gold ions. Approximately 10 g of *A. niger* biomass was transferred to a 250 mL conical flask containing distilled water (100 mL) and incubated for 72 hours at 25°C. The aqueous solution components were then separated by filtration again using filter paper. HAuCl₄ (10⁻³ M) was added to the liquid fungal solution and kept at 25°C for 72 hours. The control, fungal liquid of *A. niger* without gold chloride, was maintained separately under the same conditions.

Characterization of AuNPs

Periodically, aliquots of the reaction solutions were removed and their absorption was measured in a microplate reader (Microscan MS5608A; Electronics Corporation of India Limited, Hyderabad, India). The solution was then converted in powder for X-ray diffraction (XRD) measurements. For XRD studies, dried nanoparticles were coated on the XRD grid and the spectra were recorded by X-ray diffractometer (D-8 Advance; Bruker AXS, Madison, WI). The micrographs of AuNPs were obtained by transmission electron microscope (CM-10; Philips, Amsterdam, the Netherlands) and confirmed by scanning electron microscope JSM 5800 LV JEOL, JAPAN.

Bioassays

The larvicidal activity of AuNPs against *A. stephensi*, *C. quinquefasciatus*, and *A. aegypti* was assessed using the standard method.¹² All larvae of *A. stephensi*, *C. quinquefasciatus*, and *A. aegypti* were placed in separate containers and then placed in another container containing microbe-free deionized water. Different test concentrations of AuNPs in deionized water (100 mL) were then prepared in 250 mL beakers. Bioassays were conducted separately for each larval instar at six different log test concentrations

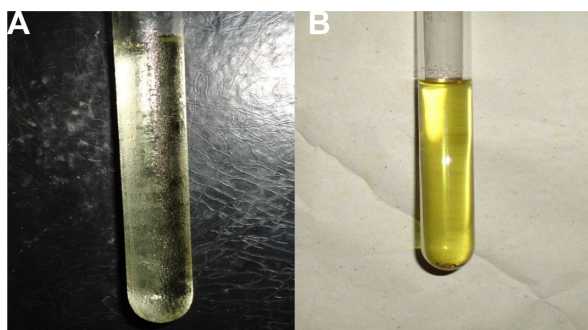


Figure 1 Test tubes containing the fungal liquid of *Aspergillus niger*: (A) control (without HAuCl₄) and (B) 72 hours after adding 10⁻³ M aqueous HAuCl₄ solution.

(0.77, 1.07, 1.25, 1.38, 1.47, and 1.55 ppm) of aqueous AuNPs. To test the larvicidal activity of AuNPs, 20 larvae from each larval instar were separately exposed to 100 mL of test concentration. Similarly, the control (without AuNPs) was run to test the natural mortality. The mortality was determined at different time points (24, 48, and 72 hours) during the treatment. Glucose powder (1 g/100 mL) was offered to the larvae during the experiments. Experiments were replicated three times to validate the results.

Data management and statistical analysis

The data on efficacy were subjected to probit analysis.¹³ The control mortality was corrected by Abbott's formula.¹⁴ The relationship between probit and log concentration was established as a probit equation and probit regression lines were drawn for each larval instar.

Results

Visual analysis of AuNPs

Figure 1A shows a test tube of *A. niger* fungal liquid before immersion in HAuCl₄ – the white color of the fungal liquid can be seen clearly. Figure 1B shows a test tube containing the fungal liquid after exposure to 10⁻³ M aqueous solution of HAuCl₄ for 72 hours – the

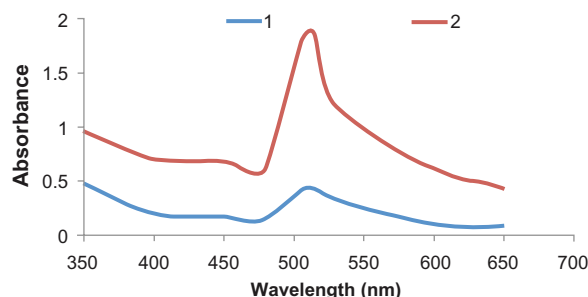


Figure 2 Microplate-recorded spectra of *Aspergillus niger* from fungal liquid before (curve 1) and after (curve 2) immersion in 10⁻³ M aqueous HAuCl₄ solution for 72 hours.

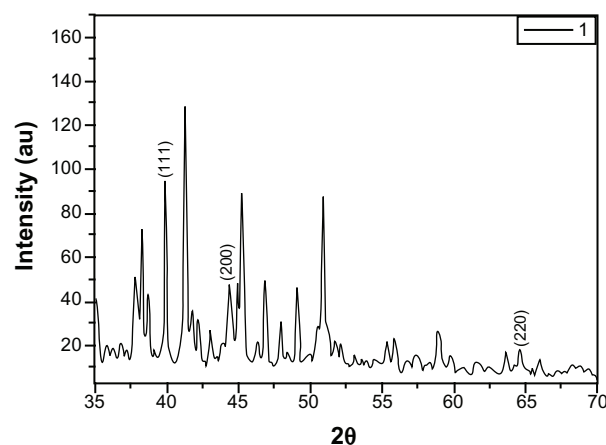


Figure 3 X-ray diffraction pattern in 2-theta (2θ) scale with counts to depict number of gold nanoparticles synthesized by *Aspergillus niger*.

yellow color seen clearly in the fungal liquid indicates the synthesis of AuNPs.

Microplate reader analysis of AuNPs

The AuNPs prepared with *A. niger* were characterized by microplate reader after incubation. Figure 2 shows the microplate-recorded spectra for *A. niger* fungal liquid before and after the addition of 10⁻³ M aqueous solution of HAuCl₄. The fungal liquid exposed to Au⁺ ions shows a distinct and fairly broad absorption band centered at 530 nm. The presence of the broad resonance indicates an aggregated structure of the gold particles in the fungal liquid.

XRD analysis of AuNPs

Figure 3 shows the XRD patterns obtained for the AuNPs synthesized by *A. niger*. The presence of the intense peaks

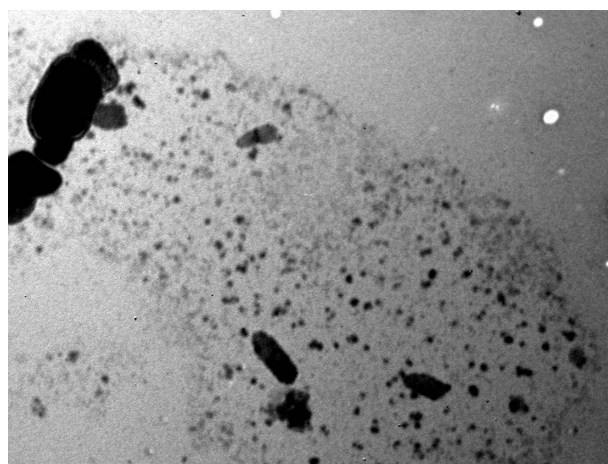


Figure 4 Transmission electron microscopy image of gold nanoparticles synthesized by *Aspergillus niger*.

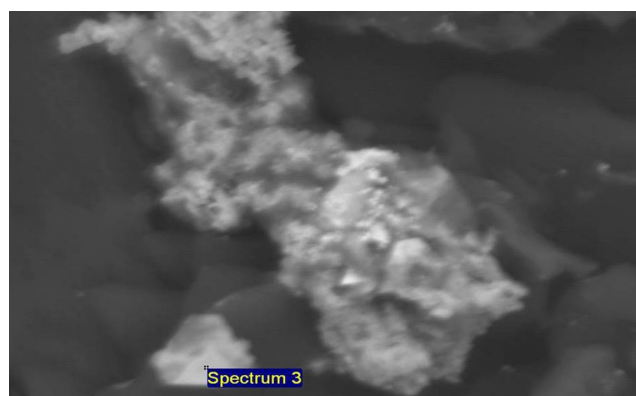


Figure 5 Scanning electron microscopy image of gold nanoparticles synthesized by *Aspergillus niger*.

(38.4°, 44.4°, and 64.2°) of the AuNPs (111), (200), and (220) appeared which are indexed as crystalline gold face-centered cubic phase. The XRD pattern clearly shows that the AuNPs formed by the reduction of AuCl_4^- ions by *A. niger* are crystalline in nature.

Transmission electron microscopy and scanning electron microscopy analysis of AuNPs

After reduction, AuNPs were precipitated at the bottom of the conical flask. This precipitate was washed out twice with twice-distilled water and then analyzed by transmission electron microscope. The samples of AuNPs synthesized using fungal liquid were prepared by placing a drop of reaction mixture on a copper grid and allowing the water to evaporate. Figure 4 shows a typical transmission electron microscopy image of AuNPs synthesized by *A. niger*. AuNPs of different sizes (10–30 nm) and shapes were observed. The scanning electron microscopy image in Figure 5 show distinctly the high-density AuNPs synthesized by *A. niger*, further confirming the development of gold nanostructures.

AuNPs synthesized by *A. niger*: efficacy against *C. quinquefasciatus* larvae

All larval instars of *C. quinquefasciatus* were found to be highly susceptible to AuNPs synthesized by *A. niger*.

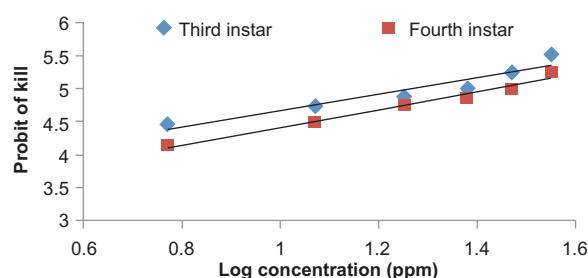


Figure 6 Relationship between probit of kill and log concentrations of *Aspergillus niger*-synthesized gold nanoparticles showing probit regression lines in larvae of *Aedes aegypti* after 72 hours.

All *C. quinquefasciatus* larvae showed 100% mortality following exposure to the AuNPs synthesized by *A. niger*. The significant mortality was recorded after 48 hours of exposure.

AuNPs synthesized by *A. niger*: efficacy against *A. aegypti* larvae

A. aegypti larval instars were found to be susceptible to AuNPs synthesized by *A. niger*. The first- and second-instar larvae were found to be highly susceptible to the AuNPs compared with the other instars. The mortality was recorded after 72 hours of exposure.

The first and second larval instars of *A. aegypti* showed 100% mortality following exposure to the AuNPs synthesized by *A. niger*. The tests were conducted at the higher selected concentrations, as the lower dose was not found to be effective. However, third (50% lethal concentration [LC_{50}], 24; 90% lethal concentration [LC_{90}], 37.15; and 99% lethal concentration [LC_{99}], 69.18 ppm) and fourth (LC_{50} , 30; LC_{90} , 45.70; and LC_{99} , 87.09 ppm) larval instars were observed with their probit equations and 95% confidence intervals after 72 hours (Table 1). The mortality rates of 0.90 and 0.97 were observed for the third and fourth larval instars, respectively. The chi-square values at 4 degrees of freedom were 13.24 and 13.94 for third, and fourth larval instars. All of the chi-square values for third and fourth larval instars of *A. aegypti* were found higher than the critical value of chi-square at a significance level of 0.05. Figure 6 shows the probit regression lines drawn for each larval instar of

Table 1 Probit equations and susceptibility of *Aedes aegypti* larvae to gold nanoparticles synthesized by *Aspergillus niger*

Larval instar	Probit equation	LC_{50} [ppm] (95% CI)	LC_{90} [ppm] (95% CI)	LC_{99} [ppm] (95% CI)
First	*	*	*	*
Second	*	*	*	*
Third	$Y = 0.18 + 3.87x$	24 (22.89–25.12)	37.15 (36.01–38.29)	69.18 (67.93–70.43)
Fourth	$Y = 0.16 + 3.68x$	30 (28.86–31.14)	45.70 (44.50–46.90)	87.09 (85.78–88.4)

Note: *100% mortality.

Abbreviations: 95% CI, 95% confidence interval; LC_{50} , 50% lethal concentration; LC_{90} , 90% lethal concentration; LC_{99} , 99% lethal concentration.

A. aegypti. No mortality could be observed in the control group. The observed LC values have shown the degree of susceptibility of fungal AuNPs among the four larval stages of *A. aegypti* in order of first > second > third > fourth larval instar.

AuNPs synthesized by *A. niger*: efficacy against *A. stephensi* larvae

The larval stages of *A. stephensi* were found to be susceptible to AuNPs synthesized by *A. niger*. The first-instar larvae were found to be more highly susceptible than the other instars. The mortality was recorded after 72 hours of exposure.

The first larval instar of *A. stephensi* showed 100% mortality when exposed to the AuNPs synthesized by *A. niger*, whereas the second (LC₅₀, 1.65; LC₉₀, 24; and LC₉₉, 30.90 ppm), third (LC₅₀, 1.69; LC₉₀, 30; and LC₉₉, 36.30 ppm), and fourth larval instars (LC₅₀, 12; LC₉₀, 39.81; and LC₉₉, 58.88 ppm) were observed with their probit equations and confidential limits after 72 hours (Table 2). Mortality rates of 0.91, 0.83, and 0.92 were observed for second, third, and fourth larval instars, respectively. The chi-square values at 4 degrees of freedom were 23.33, 22.21, and 16.6 for second, third, and fourth larval instars, respectively. All of the chi-square values for second, third, and fourth larval instars of *A. stephensi* were found to be higher than the critical value of chi-square at a significance level of 0.05. Figure 7 shows the probit regression lines drawn for each larval stage of *A. stephensi*. No mortality could be observed in the control group. The observed LC values have shown the degree of susceptibility of fungal AuNPs among the four larval stages of *A. stephensi* in order of first > second > third > fourth larval instar.

Discussion

A. niger is a filamentous, keratinophilic, and entomopathogenic fungi that has been found to be effective against mosquito larvae. In the present study the authors synthesized AuNPs with *A. niger*. Additionally, the authors investigated the larvicidal efficacy of the synthesized AgNPs against *A. stephensi*, *C. quinquefasciatus*, and *A. aegypti* larvae.

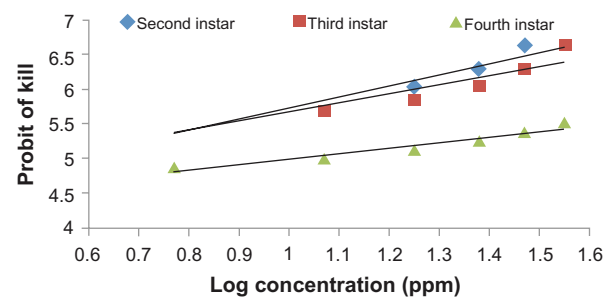


Figure 7 Relationship between probit of kill and log concentrations of *Aspergillus niger*-synthesized gold nanoparticles showing probit regression lines in larvae of *Anopheles stephensi* after 72 hours.

Fungi are well known to secrete large amounts of proteins, enzymes, toxins, and other components that play a major role in their life cycle. The process of synthesis occurs in the presence of reductase enzymes, which may be present in the cell-free extracts of *A. niger*. These enzymes are supposed to reduce the silver ions to AgNPs. However, the interaction between protein and nanoparticles is still not completely understood and requires further study.

Previously, the extracellular biosynthesis of AgNPs by using a fungus named *Trichoderma reesei* has been evaluated.¹⁵ Similarly, the biosynthesis of AgNPs using *Trichosporon beigelii* NCIM 3326 and the antimicrobial activity of these AgNPs has also been evaluated.¹⁶ Consensus has emerged that reduction of the aqueous silver ions occurs by an enzymatic process, thus indicating a possibility for the development of an eco-friendly, fungus-based nanomaterial synthesis.

Entomopathogenic fungi-synthesized AuNPs are unique because, unlike other mosquito control agents, fungal AuNPs have the ability to directly infect the host insect by penetrating the cuticle of the insect and these AuNPs do not need to be ingested by the insect to cause disease. There are preferential advantages when fungal AuNPs are used as a biocontrol agent for mosquitoes. The fungal AuNPs have a very narrow range, and considerable progress has been made in recent years in the development of environmentally benign spores and mycelium-based biocontrol agents for the mosquito population. Fungal biocontrol agents have reduced

Table 2 Probit equations and susceptibility of *Anopheles stephensi* to gold nanoparticles synthesized by *Aspergillus niger*

Larval instar	Probit equation	LC ₅₀ [ppm] (95% CI)	LC ₉₀ [ppm] (95% CI)	LC ₉₉ [ppm] (95% CI)
First	*	*	*	*
Second	$Y = 0.22 + 4.77x$	1.65 (0.21–2.99)	24 (22.91–25.09)	30.90 (29.78–32.02)
Third	$Y = 0.22 + 4.66x$	1.69 (0.35–3.03)	30 (28.91–31.09)	36.30 (35.18–37.42)
Fourth	$Y = 0.22 + 4.01x$	12 (10.88–12.12)	39.81 (38.64–40.88)	58.88 (57.65–60.11)

Note: *100% mortality.

Abbreviations: 95% CI, 95% confidence interval; LC₅₀, 50% lethal concentration; LC₉₀, 90% lethal concentration; LC₉₉, 99% lethal concentration.

inputs of harmful synthetic chemical pesticide in agriculture, horticulture, and forest systems.

The larvicidal potential of the hexane, chloroform, ethyl acetate, acetone, methanol, and aqueous leaf extracts of *Nelumbo nucifera* and of AgNPs synthesized using aqueous leaf extract against fourth-instar larvae of *A. subpictus* and *C. quinquefasciatus* have been tested.¹⁷ The larvae were exposed to varying concentrations of plant extracts and synthesized AgNPs for 24 hours. Recently, the larvicidal activity of AgNPs synthesized using *Eclipta prostrata* leaf extract against filariasis and malaria vectors has been evaluated.¹⁸ The results were based on plant-synthesized AgNPs. These AuNPs have been tested against malaria, filariasis, and dengue vector larvae.

AgNPs synthesized using the fungus *Cochliobolus lunatus* have been tested for larvicidal potential against *A. aegypti* and *A. stephensi*.¹⁹ The larvicidal potential of AgNPs synthesized by *C. lunatus* has also been tested against the nontarget fish species *Poecilia reticulata*, the most common organism in the habitats of *A. aegypti* and *A. stephensi*: there was no toxicity shown at LC₅₀ and LC₉₀ doses of the AgNPs. This indicates that nontarget organisms will not be affected, as only the mosquito larvae are targeted. In the present study, the AuNPs synthesized by *A. niger* were tested against all larval instars of *A. stephensi*, *C. quinquefasciatus*, and *A. aegypti*.

Conclusion

In the present study the AuNPs were synthesized using the keratinophilic fungus *A. niger*. The AuNPs were tested for larvicidal efficacy against *A. stephensi*, *C. quinquefasciatus*, and *A. aegypti* larvae. The AuNPs synthesized by fungus had an immediate impact on mosquito control. Therefore, the authors conclude that fungus-synthesized AuNPs may be a more rapid and environmentally friendly approach for vector control than current approaches.

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Disclosure

The authors report no conflicts of interest in this work.

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